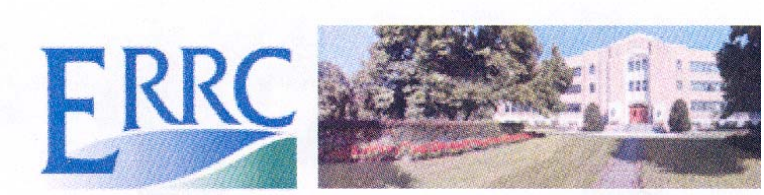

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Title: Reduction of *Escherichia coli* O157:H7 and *Salmonella* on Laboratory-Inoculated Alfalfa Seed with Commercial Citrus-Related Products

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Reduction of *Escherichia coli* O157:H7 and *Salmonella* on Laboratory-Inoculated Alfalfa Seed with Commercial Citrus-Related Products†

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ABSTRACT

Alfalfa sprouts contaminated with the bacterial pathogens *Escherichia coli* O157:H7 and *Salmonella* have been the source of numerous outbreaks of foodborne illness in the United States and in other countries. The seed used for sprouting appears to be the primary source of these pathogens. The aim of this study was to determine whether the efficacy of commercial citrus-related products for sanitizing sprouting seed is similar to that of high levels of chlorine. Five products (Citrex, Pangermex, Citricidal, Citrobio, and Environné) were tested at concentrations of up to 20,000 ppm in sterile tap water and compared with buffered chlorine (at 16,000 ppm). Alfalfa seeds were inoculated with four-strain cocktails of *Salmonella* and *E. coli* O157:H7 to give final initial concentrations of ca. 9.0 and 7.0 CFU/g, respectively. Treatments (10 min) with Citrex, Pangermex, and Citricidal at 20,000 ppm and chlorine at 16,000 ppm produced similar log reductions for alfalfa seed inoculated with four-strain cocktails of *E. coli* O157:H7 and *Salmonella* (3.42 to 3.46 log CFU/g and 3.56 to 3.74 log CFU/g, respectively), and all four treatments were significantly ($P < 0.05$) more effective than the control treatment (a buffer wash). Citrobio at 20,000 ppm was as effective as the other three products and chlorine against *Salmonella* but not against *E. coli* O157:H7. Environné was not more effective (producing reductions of 2.2 to 2.9 log CFU/g) than the control treatment (which produced reductions of 2.1 to 2.3 log CFU/g) against either pathogen. None of the treatments reduced seed germination. In vitro assays, as well as transmission electron microscopy, confirmed the antibacterial nature of the products that were effective against the two pathogens and indicated that they were bactericidal. When used at 20,000 ppm, the effective citrus-related products may be viable alternatives to chlorine for the sanitization of sprouting seed pending regulatory approval.

Alfalfa sprouts contaminated with the bacterial pathogens *Escherichia coli* O157:H7 and various serovars of *Salmonella* have been implicated in numerous outbreaks of foodborne illness since 1995 both in the United States and in other countries *(26)*. On the basis of epidemiological evidence as well as direct isolation, the seed used for sprouting appears to be the primary source of these pathogens *(2, 17, 18, 20)*. There are numerous published reports on the efficacies of various volatile and nonvolatile chemicals for reducing or eliminating bacterial pathogens from laboratory-inoculated seed. The challenge is to eliminate the pathogen while maintaining seed viability and sprout yield and quality. If pathogens are not eliminated, even low levels of survivors can grow to high levels on growing sprouts owing to favorable environmental and nutritional conditions during the sprouting process *(23)*.

Chemicals tested previously include NaOCl, $Ca(OCl)_2$, $Ca(OH)_2$, H_2O_2, CH_3CH_2OH, Na_3PO_4, acidified ClO_2, acidified $NaClO_2$, various essential oils and organic acids, ammonia, ozonated water, and a variety of commercial antimicrobial washes, sometimes in combination with heat *(1, 9, 11, 12, 19, 21, 22, 25, 33, 34)*. Some chemical treatments have been found to be very effective in reducing or eliminating *E. coli* O157:H7 and *Salmonella* from alfalfa seed but highly detrimental to seed germination. To date, none of the chemical treatments investigated have successfully eliminated bacterial human pathogens from laboratory-inoculated alfalfa seed while maintaining an acceptable level of seed germination.

Currently, the U.S. Food and Drug Administration and the California Department of Health Services recommend that sprout growers treat their seed with 20,000 ppm of free chlorine from $Ca(OCl)_2$ (or with an equivalent approved antimicrobial treatment) for 15 min in addition to testing spent irrigation water for the presence of *Salmonella* and *E. coli* O157:H7 *(28, 29, 31)*. This recommendation, along with the fact that treatment with chlorine at concentrations of up to 20,000 ppm is currently the only chemical treatment approved by the U.S. Environmental Protection Agency for use on sprouting seed, poses a challenge for organic sprout growers who are not allowed by certifying organizations to treat seed with such a high level of chlorine and still sell an organic product. Effective natural antimicrobial agents are highly desirable. In this study, we wished to determine whether various citrus-related commercial products are as effective as high levels of chlorine in the reduction of bacterial pathogens on laboratory-inoculated al-

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† Mention of a brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

falfa seed. If so, such products would be useful to commercial organic growers as well as to those who grow sprouts in their homes for personal consumption.

MATERIALS AND METHODS

Bacterial strains and seed. The strains of *E. coli* O157:H7 used in this study were strains F4546 (a clinical strain associated with a sprout-related outbreak that occurred in Michigan and Virginia in 1997), SEA13B88 (isolated from apple cider implicated in an outbreak in the western United States in 1996), C7927 (a clinical strain associated with an apple cider–related outbreak in Massachusetts in 1992), and Ent-C9490 (a clinical strain associated with a ground beef–related outbreak in the western United States in 1993). All strains except F4546 were obtained from Dr. Pina Fratamico (U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center). Strain F4546 was obtained from Dr. Robert Buchanan (U.S. Food and Drug Administration). *Salmonella* Anatum F4317, *Salmonella* Infantis F4319, *Salmonella* Newport H1275, and *Salmonella* Stanley H0558 were obtained from Dr. Patricia Griffin (Centers for Disease Control and Prevention, Atlanta, Ga.). All four strains were implicated in sprout-related outbreaks in the United States. No inhibition among the four strains of *Salmonella* or *E. coli* O157:H7 was noted when the strains were streaked perpendicular to one another on tryptic soy agar (TSA; Difco Laboratories, Detroit, Mich.) and incubated for 24 h at 37°C. For long-term storage, strains were kept at −80°C in tryptic soy broth (TSB; Difco) plus 20% glycerol.

Alfalfa seed was purchased from Caudill Seed Company, Inc. (Louisville, Ky.). The native microflora was eliminated by exposing seed in stomacher bags with mesh liners to 25 kGy of gamma irradiation. Sterility was confirmed by enrichment. Irradiated seed (10 g) was placed in a sterile stomacher bag, and then 40 ml of TSB was added and the bag's contents incubated at 30°C for 20 h. After incubation, four 0.25-ml samples were plated on TSA plates. Plates were incubated at 30°C for 48 h and then examined for growth.

Seed inoculation. To prepare inoculum, bacterial strains were streaked from frozen stock cultures onto TSA, and plates were incubated for 18 h at 37°C. A single colony was used to inoculate TSB (3 ml), with one tube being used for each strain. The broth cultures were incubated for 18 h in a shaking incubator (250 rpm) at 37°C. For *E. coli* O157:H7, 0.1-ml starter cultures were used to inoculate 25 ml of TSB with 1.0% (wt/vol) dextrose (Difco) (supplemented with additional dextrose at 7.5 g/liter) to induce pH-dependent stationary-phase acid resistance *(3, 4)*. The broth media were kept in 125-ml flasks (two flasks per strain) and incubated statically at 37°C for 18 h. The same procedure was used for *Salmonella* except that TSB was not supplemented with additional dextrose and the broth cultures were shaken at 250 rpm. Preliminary experiments indicated that the four strains of *E. coli* O157:H7 attained populations of approximately 1×10^9 CFU/ml, while the four strains of *Salmonella* attained populations of approximately 9×10^9 CFU/ml. After incubation, the contents of the eight flasks were combined in a single sterile 500-ml flask and mixed, and then the mixture of strains was transferred to sterile centrifuge bottles. Bacterial cells were pelleted by centrifugation, the pellets were washed once with sterile 0.1% (wt/vol) peptone (Difco) water (PW), the washed cells were recentrifuged, and the final cell pellets were resuspended in 250 ml of PW. The cell concentration in the final inoculum was determined by preparing decimal dilutions in PW and plating them in duplicate on TSA (0.1 ml per plate). Colonies were counted after incubation for 18 h at 37°C. For the inoculation of alfalfa seed, 50 ml of the inoculum cocktail was added to each of five stomacher bags containing 100 g of sterile alfalfa seed per bag. The bags were massaged by hand for 1 min, and excess inoculum was decanted. The mesh liners containing the inoculated seed were placed under a biosafety cabinet on a sterile tray covered with wire mesh and allowed to dry for 48 h. To determine the initial inoculum load on the seed, 20 ml of buffered PW (Difco) was added to a sterile stomacher bag containing 10 g of inoculated seed. The seed was pummeled for 1 min in a stomacher (Seward, normal setting), and then decimal dilutions of the homogenate were prepared with PW. For *E. coli* O157:H7, dilutions were plated in triplicate on TSA, eosin methylene blue agar (Difco) (0.1 ml per plate), and Petrifilm *E. coli*/coliform and *Enterobacteriaceae* count plates (3M) (1.0 ml per plate). For *Salmonella,* dilutions were plated in triplicate on TSA and XLT 4 (Difco). Colonies were counted after 24 and 48 h of incubation at 37°C. Dried, inoculated seed was placed in sterile stomacher bags and stored at 4°C until it was used. Experiments were completed within 2 months of seed inoculation. No changes in the populations of the pathogens on the inoculated seed during this period were noted.

Chemicals and seed treatments. Citrex concentrate (100%), a highly viscous liquid, was supplied by Citrex, Inc. (Miami, Fla.). This product consists of a mixture of organic acids, including citric acid and ascorbic acid, a fatty acid, a natural antioxidant, and sugars (Citrex, personal communication). The product's literature suggests that the product is antimicrobial. Citrex is the active ingredient in two products sold by Citrex, Inc.: LonLife and Bio-Clean. LonLife is sold as an organic wide-spectrum antimicrobial agent for the control of various bacterial, fungal, and viral pathogens of agricultural crops. Bio-Clean is sold as an organic wide-spectrum disinfectant.

Pangermex concentrate (100%), a highly viscous liquid, was supplied by Ascedar Industria e Comércio LTDA, S. J. Campos, Brazil. This product is an extract from oranges in which the ascorbic acid content is adjusted to a final concentration of 5% (Ascedar, personal communication). Pangermex is also available in powdered form.

Citricidal, a highly viscous liquid concentrate, was purchased from NutriTeam, Inc. (Ripton, Vt.). According to its label, this product consists of grapefruit seed and pulp extractives (60%) and vegetable glycerin (40%). No product usage rates are given on the label, but it is directed that the product be diluted. Literature from the manufacturer states that the product has antibacterial, antifungal, and antiviral activities.

Citrobio CB20, a nonviscous liquid, was purchased from Citrobio, Inc. (Sarasota, Fla.). According its label, this product is an antibacterial wash for fruits and vegetables, seafood and shrimp, and poultry and meat and consists of citric acid (0.4%), water, oranges, glycerin, and tocopherols. The recommended usage rate is ca. 2,000 ppm (7.4 ml/3.79 liters) with submersion for 5 to 10 min. Citrobio's label indicates that the product is acceptable for organic use according to the Organic Materials Review Institute.

Environné Fruit and Vegetable Wash, manufactured by Consumer Health Research, Inc. (Bandon, Oreg.), was purchased from a local supermarket. According to the product's label and literature, this "all natural product" consists of nonionic and anionic surfactants (derived from natural plant oils), polysorbate-20 (derived from sorbitol), grapefruit seed extract, and lemon and orange extract. Environné is sold as a wash designed to remove chemical residues and waxes. The product's directions suggest applying a small amount directly to produce, massaging for approximately 30 s, and then rinsing with water. The recommended usage rate for "hard-to-clean produce" (with grapes, broccoli, and mushrooms cited as examples) is approximately 100,000 ppm (3 tablespoons per pint of

water), and agitation for 1 min with a vegetable brush, if necessary, is suggested. Solutions were prepared immediately prior to use. No claims of antimicrobial activity are listed on the label.

In our experiments, the various products were tested at 1,000, 5,000, 10,000, and 20,000 ppm and prepared with either sterile tap water or 500 mM potassium phosphate buffer (pH 6.8). For comparison, chlorine solutions were prepared with $Ca(OCl)_2$ (3.0%, wt/vol, 65% active chlorine; Aldrich Chemical Company, Milwaukee, Wis.) and with sterile 500 mM potassium phosphate buffer (with a final pH of 6.8). Chlorine solutions were stirred for 20 min at room temperature before they were used, and their free chlorine contents were determined after appropriate dilution with purified water (18.2 MΩ-cm resistivity) with the use of an Environmental Protection Agency–approved commercial test kit (Accuvac, Hach Company, Loveland, Colo.) based on the *N,N*-diethyl-*p*-phenylenediamine method.

For each seed treatment, 10 g of inoculated seed was placed in a sterile stomacher bag and rinsed twice by the addition of 50 ml of sterile tap water to the stomacher bag followed by agitation by hand for 2 min for each wash. Fifty milliliters of the sanitizer solution (or buffer alone as a control) was then added, and the bag's contents were agitated by hand for 10 min. After the decanting of the sanitizer solution, the treated seed was rinsed twice with sterile tap water as described above. After the final rinse, 20 ml of D/E neutralizing broth (Difco) was added and the suspension was pummeled in a stomacher as described above. Serial decimal dilutions were prepared from the homogenates with sterile PW and undiluted homogenate, and serial dilutions were plated in triplicate on Petrifilm *E. coli*/coliform count plates (1.0 ml per plate) and on TSA (0.1 ml per plate) (for *E. coli* O157:H7) or on XLT 4 and TSA (0.1 ml per plate) (for *Salmonella*). Even though *E. coli* O157:H7 strains do not produce β-glucuronidase, *E. coli* Petrifilm was used as a selective medium for the enumeration of injured cells (cells that grow out on nonselective media but not on selective media). The inability to differentiate colonies of coliforms from those of *E. coli* O157:H7 on this medium (both types of colonies were red with gas) was not an issue in these experiments because seeds were sterilized before inoculation. Plates were incubated at 37°C, and final CFU counts were obtained after 24 to 48 h. Two random colonies per TSA plate were confirmed to be *E. coli* O157:H7 or *Salmonella* by a commercial latex agglutination test (RIM, Remel, Lenexa, Kans.) or by slide agglutination with *Salmonella* O antisera Poly A-I and Vi (Difco), respectively. As a further control, unrinsed and unsanitized seed was pummeled in the presence of D/E neutralizing broth, and bacterial populations were determined as described above. The rinsing of seed was carried out both before and after chemical treatment to mimic common commercial practice.

Seed germination tests. Nonirradiated seed samples (1 g each) were placed in 20-ml glass beakers. Seeds were rinsed twice with 5 ml of sterile tap water for 2 min with mechanical stirring. Next, 5 ml of commercial product solution prepared with sterile tap water was added, and the suspension was stirred mechanically for 10 min. After the decanting of the solution, seeds were again rinsed twice with sterile water as described above. Seeds were then soaked for 2 to 3 h in sterile tap water. After soaking, 100 seeds for each treatment were transferred to sterile glass culture dishes lined with wetted filter paper and left to germinate on a lab bench at room temperature for 2 days. Seeds with emerging roots that were visible to the naked eye were considered germinated. Seeds that were rinsed and treated for 10 min with sterile water as described above were germinated as controls. The germination experiments were carried out four times.

In vitro antibacterial assays. The MICs of the five citrus-related products were determined for *Salmonella* Stanley H0558 and *E. coli* O157:H7 strain F4546 by a macro broth dilution method according to Washington and Barry *(32)*. The bacteria were first cultured overnight on TSA at 37°C. For each bacterium, an isolated colony was transferred to 25 ml of TSB in a 250-ml flask. Flasks were shaken (250 rpm) at 37°C for 18 h. The bacterial suspensions (10^9 to 10^{10} CFU/ml) were diluted 1:2,000 in double-strength TSB. Each citrus-related product was diluted in sterile tap water to twice the highest final concentration desired. Serial twofold dilutions in sterile plastic culture tubes (12 by 75 mm) were prepared with sterile tap water as the diluent, and each tube contained a volume of 1 ml after dilution. The final tubes (control tubes) for each dilution series contained 1 ml of sterile water only. One milliliter of inoculum was then added to each tube, and the tubes were kept at 37°C without shaking for 16 to 20 h. After incubation, the lowest concentration of citrus-related product resulting in the complete inhibition of growth visible to the naked eye represented the MIC. The experiment was carried out three times.

To determine whether the citrus-related products exhibited bactericidal activity, *Salmonella* Stanley H0558 and *E. coli* O157:H7 were grown overnight on TSA at 37°C. A single isolated colony was used to inoculate 25 ml of TSB into a 250-ml flask (one flask per strain). Cultures were incubated overnight at 37°C with shaking (250 rpm). Cultures were then transferred to 50-ml sterile conical centrifuge tubes, and cells were pelleted by centrifugation and then washed with sterile tap water. After recentrifugation, the cells were washed for a second time. The final cell pellets were suspended in 25 ml of sterile tap water. Solutions (40,000 ppm) of each commercial product were prepared with sterile tap water. Three milliliters of each commercial product was added to 3 ml of bacterial suspension in a sterile plastic culture tube, mixed well, and left to sit for 10 min at room temperature. The suspensions were then mixed well, and then dilutions were prepared with D/E broth. Undiluted or diluted samples were plated on TSA and the selective medium XLT 4 (for *Salmonella*) or *E. coli* Petrifilm (for *E. coli* O157:H7) (three plates per dilution, 100 μl per plate). Plates were incubated for 24 to 48 h at 37°C, and survivors were enumerated. For controls, sterile tap water was used instead of a commercial product.

Microscopy. *Salmonella* Stanley H0558 and *E. coli* O157:H7 strain F4546 were cultured, and the cells were harvested and washed and then treated with Citrex (20,000 ppm, 10 min) as described above for the in vitro antibacterial assays. After treatment with Citrex, the samples were recentrifuged and the cells were washed twice with sterile tap water. The final cell pellets were taken up in 25 ml of sterile tap water. For the controls, the 10-min treatments were carried out with sterile tap water rather than with the Citrex solution. Cell suspensions (900 μl each) were added to 1.5-ml plastic microcentrifuge tubes, and 100 μl of 25% glutaraldehyde (Electron Microscopy Services, Fort Washington, Pa.) in 0.1 M imidazole buffer (pH 6.8) was added. After mixing, the samples were centrifuged in a microcentrifuge to pellet the cells. The supernatant fluids were removed, 200 μl of molten 2% agarose was added to each tube, and the tube contents were mixed well and then recentrifuged in a microcentrifuge. After agarose had solidified, the tips of the tubes were cut off below the agarose pellets and the tubes were placed in glass vials containing 10 ml of 2.5% glutaraldehyde in buffer. The samples were stored at room temperature overnight, and then the pellets were removed from the tubes with forceps and washed in 0.1 M imidazole buffer (pH 6.8).

Secondary fixation was carried out with 2% osmium tetroxide in 0.1 M imidazole buffer (pH 6.8) for 2 h, and the samples

were then washed with distilled water. Dehydration was carried out with a graded series of ethanol concentrations (50%, 80%, and absolute). The dehydrated samples were infiltrated with a 1:1 mixture of propylene oxide and epoxy resin and cured for 2 days at 55°C. Thin sections were cut with diamond knives, stained with 2% uranyl acetate and lead citrate solutions, and imaged photographically with a model CM12 electron microscope (FEI/Philips, Hillsboro, Oreg.) operated in the bright field mode.

Statistical analysis. For statistical analysis, a completely randomized design was used, and data were analyzed by analysis of variance to determine the effects of treatments on the log reduction responses. Mean separations were analyzed for significant differences ($P < 0.05$) by the least significance difference separation procedure *(16)*.

RESULTS

Chemical treatment of seed. The irradiated alfalfa seed was confirmed to be sterile on the basis of a lack of microbial growth after broth enrichment and plating on TSA. The use of inoculated sterile seed allowed the use of both selective and nonselective agar media for distinguishing injured and noninjured bacterial cells after chemical treatment. Inoculum cocktails contained 8.47×10^9 to 9.27×10^9 CFU/ml for *Salmonella* and 8.60×10^8 to 1.61×10^9 CFU/ml for *E. coli* O157:H7. Populations on inoculated seed ranged from 8.58 to 9.01 $\log_{10}$ CFU/g for *Salmonella* and from 6.72 to 7.12 $\log_{10}$ CFU/g for *E. coli* O157:H7. *E. coli* O157:H7 cells induced to pH-dependent stationary-phase resistance *(3, 4)* were used because bacteria in their natural environments may be exposed to a variety of environmental stresses, including dehydration and nutrient depravation. Similar acid-adapted cells of the *Salmonella* strains were not used for inoculation because acid-adapted cells of *Salmonella* Typhimurium have been reported to have increased sensitivity to hypochlorous acid *(13)*. Plating on selective and nonselective agar media revealed no significant populations of injured *Salmonella* or *E. coli* O157:H7 cells on the inoculated seed.

When tested on alfalfa seed inoculated with *E. coli* O157:H7, the concentrates Citrex and Pangermex, as well as Citricidal at 20,000 ppm in sterile tap water, produced the largest log reductions (3.42 to 3.66 $\log_{10}$ CFU/g); their efficacies were similar to that of 16,000 ppm of free chlorine in buffer (at a final pH of 6.8) (Table 1). All four treatments were significantly ($P < 0.05$) more effective than the control treatment (rinsing plus buffer treatment) (Table 1). Because of the low pHs of Citrex and Pangermex solutions at the higher concentrations, these two products were also prepared with buffer (with a final pH of 6.8) at concentrations of up to 10,000 ppm. Buffered solutions were as active as unbuffered solutions, indicating that low pH was not responsible for their activity. Citrobio and Environné in sterile tap water at concentrations of up to 20,000 ppm were not significantly more effective than the control treatment. Similar results were obtained for alfalfa seed inoculated with *Salmonella*, except that in addition to Citrex, Pangermex, and Citricidal, Citrobio at 20,000 ppm in sterile tap water was significantly more effective than the control treatment (Table 2). None of the treatments induced cell injury for *E. coli* O157:H7 and *Salmonella* on the basis of results obtained with selective media (Tables 1 and 2).

Effects of chemical treatments on seed germination. There were no significant differences ($P < 0.05$) between the extent of the germination of alfalfa seed treated with 20,000 ppm of each citrus-related product prepared in sterile tap water and that of the seed subjected to the control treatment (Table 3).

Antimicrobial activity in vitro. On the basis of the macro broth dilution assays run with TSB, the MICs for Citrex, Pangermex, Citricidal, Citrobio, and Environné against *E. coli* O157:H7 strain F4546 were 25, 25, 100, 250, and >1,000 ppm, respectively. The corresponding MICs against *Salmonella* Stanley H0558 were 50, 100, 100, 1,000, and >1,000 ppm. The highest concentration tested in the macro broth dilution assays was 1,000 ppm.

To determine whether the citrus-related products had bactericidal activity, each was tested at 20,000 ppm against one strain of *Salmonella* and one strain of *E. coli* O157:H7. Bacteria were suspended in sterile tap water and exposed to each product for 10 min. The results obtained indicated that all citrus-related products except Envrionné were bactericidal to the two pathogens (Table 4). For the active products, the largest log reductions were produced by Citrex, Pangermex, and Citricidal, followed by Citrobio.

Results obtained with transmission electron microscopy for *E. coli* O157:H7 strain F4546 and *Salmonella* Stanley H0558 treated with Citrex (20,000 ppm, 10 min) in vitro indicated that the product had similar effects on the ultrastructures of both bacteria. The treated bacterial cells exhibited a coagulated cytosol compared with control cells (Fig. 1). Electron-dense areas in the treated cells most likely represent aggregates of disorganized ribosomes. There was no evidence of bacterial cell lysis.

DISCUSSION

Organic sprout growers require an alternative to high levels of chlorine for the sanitization of sprouting seed. Natural antimicrobial chemicals might be one such alternative. In this study, we examined the efficacy of five commercial citrus-related products for the sanitization of sprouting seed. Citrex is the concentrated active ingredient used to formulate two commercial products, while Pangermex, Citricidal, Citrobio, and Environné are sold as ready-to-use products. All of these products except Environné are claimed to have antimicrobial activity by their manufacturers. Both Citricidal and Environné contain grapefruit seed extract.

The antimicrobial activity and potential uses of grapefruit seed extract have been the subject of several studies. The spraying of *Salmonella* Typhimurium–inoculated chicken skin with 0.5% grapefruit seed extract significantly reduced the population of the pathogen (CFU/g) by 1.6 to 1.8 $\log_{10}$ *(35)*. The coating of polyethylene films used to package ground beef with 1.0% grapefruit seed extract led to reduced growth rates for both aerobic and coliform bacteria *(10)*. Treatment of citrus fruit with grapefruit seed extract at 500 ppm was highly effective in preventing mold

TABLE 1. *Reductions of* Escherichia coli *O157:H7 on inoculated alfalfa seed by treatment with commercial citrus-related products*

Treatment[a]	Concn of product (ppm)	pH	Reduction of *E. coli* O157:H7 (log CFU/g) on[b]: TSA	Petrifilm
Sterile buffer (SB)[c]	—	6.8	2.1 DE	1.6 F
Citrex in sterile water	1,000	7.4	2.3 BCDE	2.0 CDEF
	5,000	5.5	2.5 BCDE	2.1 BCDEF
	10,000	5.0	3.1 ABCD	2.8 ABCDEF
	20,000	3.9	3.4 AB	3.3 AB
Citrex in SB	1,000	6.8	2.0 E	1.8 EF
	5,000	6.8	2.2 DE	1.7 F
	10,000	6.8	3.3 ABC	3.2 ABC
Pangermex in sterile water	1,000	7.5	2.6 ABCDE	2.2 ABCDEF
	5,000	6.3	2.6 ABCDE	2.2 ABCDEF
	10,000	4.9	2.8 ABCDE	2.6 ABCDEF
	20,000	3.9	3.4 AB	3.0 ABCDE
Pangermex in SB	1,000	6.8	1.9 E	1.8 EF
	5,000	6.8	2.3 CDE	1.8 EF
	10,000	6.8	2.6 ABCDE	2.4 ABCDEF
Citricidal in sterile water	1,000	7.9	2.6 ABCDE	2.0 DEF
	5,000	7.3	2.9 ABCDE	2.3 ABCDEF
	10,000	5.8	3.1 ABCD	2.9 ABCDEF
	20,000	4.8	3.7 A	3.4 A
Citrobio in sterile water	1,000	8.1	2.3 BCDE	1.9 EF
	5,000	7.9	2.3 CDE	1.8 EF
	10,000	7.7	2.4 BCDE	2.0 DEF
	20,000	7.1	2.5 BCDE	2.1 CDEF
Environné in sterile water	1,000	8.2	2.2 CDE	1.8 EF
	5,000	8.4	2.1 DE	1.7 F
	10,000	8.5	2.3 BCDE	2.0 EF
	20,000	8.4	2.2 CDE	1.7 F
Chlorine in SB	16,000	6.8	3.3 ABC	3.2 ABCD

[a] Seed was rinsed twice with sterile water before and after each 10-min treatment.
[b] Mean for three separate experiments. Means with the same letter in the same column are not significantly different ($P < 0.05$).
[c] 500 mM potassium phosphate (pH 6.8).

caused by *Penicillium* sp. *(8)*. The quality of a variety of fruits and vegetables treated with 250 to 500 ppm of grapefruit seed extract was improved during storage at 10 to 15°C *(7)*. Treatment of lettuce and strawberries with 400 ppm of grapefruit seed extract for 10 min resulted in a reduction in total mesophilic aerobes of up to 2.4 $\log_{10}$ CFU/g *(14)*. Treatment of alfalfa sprouts with Citricidal (100 ppm) for 5 min reduced native coliforms by 1 to 2 log CFU/g and reduced *Salmonella* Typhi and *Vibrio cholerae* by up to 1.5 log CFU/g *(5)*. The antimicrobial nature of grapefruit seed extracts has come into question, however. Woedtke et al. *(30)* tested six commercial preparations of grapefruit seed extract for the presence of the antimicrobial preservatives benzethonium chloride, triclosan, and methyl parabene. Only one of the six preparations was found to be free of all three preservatives, and this preparation had no antimicrobial activity. We did not test the sample of Citricidal used in our studies for the presence of preservatives, but the manufacturer claims the product does not contain such substances.

In most instances, at the highest concentration tested in our study (20,000 ppm), four of the five citrus-related products (Citrex, Pangermex, Citricidal, and Citrobio) were as effective as high levels of chlorine (16,000 ppm) in reducing populations of *E. coli* O157:H7 and *Salmonella* on inoculated seed. The one exception was Citrobio, which was not more effective than the control treatment in reducing the level of *E. coli* O157:H7. The antibacterial nature of the four active products against the two pathogens was confirmed by in vitro assays. MICs ranged from 25 to 1,000 ppm, depending on the product and the bacterium tested. The four effective products proved to be bactericidal when tested against the two pathogens in suspension at 20,000 ppm for 10 min (a time and a concentration similar to those for the seed treatments). The one product with no claims of antimicrobial activity, Environné, did not demonstrate bacteriostatic or bacteriocidal activity against the two pathogens, even though the product label indicates that it contains grapefruit seed extract (whose concentration is not given). This product was also not effective in sanitizing inoculated alfalfa seed.

The in vitro antimicrobial activity of the four effective

TABLE 2. *Reductions of* Salmonella *serovars on inoculated alfalfa seed by treatment with commercial citrus-related products*

Treatment[a]	Concn of product (ppm)	pH	Reduction of *Salmonella* (log CFU/g) on[b]: TSA	XLT 4
Sterile buffer (SB)[c]	—	6.8	2.3 F	2.0 D
Citrex in sterile water	1,000	7.4	3.0 BCDEF	2.7 CD
	5,000	5.5	3.0 ABCDEF	2.6 CD
	10,000	5.0	3.2 ABCDE	2.6 CD
	20,000	3.9	3.6 AB	4.0 AB
Pangermex in sterile water	1,000	7.5	3.0 ABCDEF	2.6 CD
	5,000	6.3	3.2 ABCDE	2.8 BCD
	10,000	4.9	3.3 ABCD	2.8 ABCD
	20,000	3.9	3.7 A	4.2 A
Citricidal in sterile water	1,000	7.9	2.7 CDEF	2.1 D
	5,000	7.3	2.8 CDEF	2.2 D
	10,000	5.8	3.0 ABCDEF	2.4 CD
	20,000	4.8	3.6 AB	3.7 ABC
Citrobio in sterile water	1,000	8.1	2.7 CDEF	2.0 D
	5,000	7.9	2.8 BCDEF	2.3 CD
	10,000	7.7	2.8 BCDEF	2.4 CD
	20,000	7.1	3.3 ABCD	3.3 ABCD
Environné in sterile water	1,000	8.2	2.5 EF	2.3 D
	5,000	8.4	2.6 DEF	2.3 CD
	10,000	8.5	2.6 DEF	2.3 CD
	20,000	8.4	2.9 BCDEF	2.8 ABCD
Chlorine in SB	16,000	6.8	3.4 ABC	3.3 ABCD

[a] Seed was rinsed twice with sterile water before and after each 10-min treatment.
[b] Mean for three separate experiments. Means with the same letter in the same column are not significantly different ($P < 0.05$).
[c] 500 mM potassium phosphate (pH 6.8).

citrus-related commercial products was much more extensive than the activity demonstrated against the two pathogens on alfalfa seed. Most likely, this result was due to the presence of the pathogens in inaccessible locations on or in the seed, such as wrinkles *(6),* cracks in the seed coat (testa), and natural openings such as the hilum and micropyle *(27).* Bacterial pathogens present in natural openings such as stomata or internalized in cut tissues have been demonstrated to be more resistant to chlorine treatments than bacterial cells present on the surfaces of plant tissues *(24).*

To our knowledge, the specific sites of antibacterial action for the four active citrus-related products are not known, but on the basis of their multicomponent makeups, these products, like most biocides when used at high concentrations, probably target multiple sites within the bacterial cell, and damage to these sites leads to their bactericidal effects *(15).* In our study, microscopy for cells of *E. coli* O157:H7 strain F4546 and *Salmonella* Stanley H0558 treated with Citrex (20,000 ppm, 10 min) indicated that normal cytoplasmic organization was severely compromised. The cytoplasmic organization of *Bacillus subtilis* cells treated with a much lower concentration of grapefruit seed extract (100 ppm) was also similarly severely disrupted *(7).*

The results obtained in the present study indicate that

TABLE 3. *Effects of citrus-related commercial products (at 20,000 ppm in sterile water) on germination of treated alfalfa seed*[a]

Treatment	pH	% germination on day 1: Trial A	Trial B	Trial C	Trial D	Average ± SD[b]	% germination on day 2: Trial A	Trial B	Trial C	Trial D	Average ± SD[b]
Citrex	3.9	75	80	60	68	71 ± 9	85	90	64	73	76 ± 14
Pangermex	3.9	64	66	69	65	66 ± 2	79	84	76	73	78 ± 5
Citricidal	4.8	78	72	70	68	72 ± 4	87	87	74	78	82 ± 9
Citrobio	7.1	81	66	65	67	70 ± 8	91	76	76	78	80 ± 7
Environné	8.4	75	71	65	63	69 ± 6	86	91	73	72	81 ± 9
Sterile water	6.8	74	77	73	71	74 ± 3	83	87	80	75	81 ± 5

[a] For each trial, 100 seeds per treatment were germinated. Treatments lasted 10 min.
[b] Averages were not significantly different ($P < 0.05$) for this day.

TABLE 4. *Effects of 10 min of exposure to commercial citrus-related products (20,000 ppm) on viability of* Salmonella *and* E. coli *O157:H7 suspended in sterile tap water*[a]

Bacterium	Treatment	Survivor count (CFU/ml)		Reduction (log CFU/ml)	
		TSA	XLT 4/Petrifilm	TSA	XLT 4/Petrifilm
Salmonella Stanley H0558	Citrex	—[b]	—	>9.5	>9.3
	Pangermex	—	—	>9.5	>9.3
	Citricidal	—	—	>9.5	>9.3
	Citrobio	1.36	0.97	8.2	8.4
	Environné	9.58	9.50	0	0
	Sterile tap water	9.51	9.37		
E. coli O157:H7 F4546	Citrex	—	—	>9.4	>9.2
	Pangermex	—	—	>9.4	>9.2
	Citricidal	—	—	>9.4	>9.2
	Citrobio	1.67	1.75	7.8	7.5
	Environné	9.35	9.25	0.1	0
	Sterile tap water	9.45	9.25		

[a] The selective media used were XLT 4 (for *Salmonella*) and *E. coli* Petrifilm (for *E. coli* O157:H7). Values shown are the means for three separate experiments.
[b] —, <7 CFU/ml.

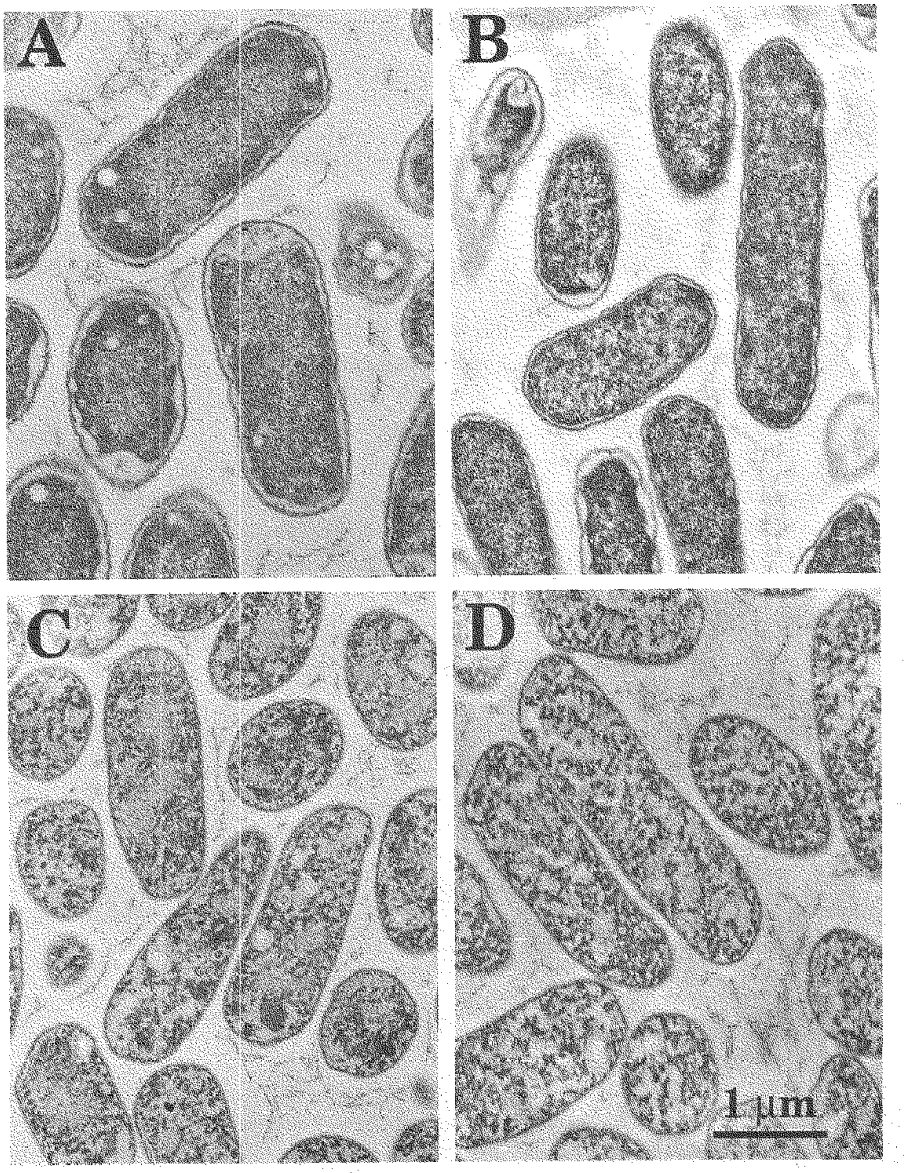

FIGURE 1. *Transmission electron microscopy for* E. coli *O157:H7 strain F4546 (A, C) and* Salmonella *Stanley H0558 (B, D) treated in vitro with sterile tap water alone (A, B) or with Citrex at 20,000 ppm for 10 min (C, D).*

the use of citrus-related commercial products for the sanitization of sprouting seed may be a viable alternative to the use of high levels of chlorine, but this alternative approach will require appropriate regulatory approval.

Addendum. After acceptance of the manuscript, the authors learned that the product Pangermex sold in Brazil is a concentrated form of the product Citrobio CB20 (a 1:20 dilution of the concentrate) sold in the United States.

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